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Development of a Recombinant Bacterial Expression System for the Active Form of a Human Transforming Growth Factor β Type II Receptor Ligand Binding Domain

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Expression systems have been designed to test the suitability of expressing the high cysteine containing extracellular domain (residues 1–136) of human transforming growth factor β type II receptor (TβRII). Receptor expressed using a baculovirus system was functional following both enzymatic deglycosylation and elimination of the N-terminal 22 amino acids by protease degradation. Bacterial expression of a TβRII lacking the 26 N-terminal amino acids retained the ability to bind its ligand, TGF-β1. Receptor expressed in bacteria was sensitive to proteolytic degradation at residue Lys98 but a K98T mutation eliminated degradation and did not disrupt binding. Although several different forms of TβRII were expressed, only a fusion with glutathione *S*-transferase gave soluble TβRII, which was purified at a yield of 0.1 mg/10 L of bacterial growth. N-Terminal truncations of TβRII (residues 22–136 or 27–136) could be refolded from inclusion bodies and purified to an active form with an efficiency of 10%.

Transforming growth factor β (TGF-β)[2] is found in most eukaryotic organisms, including *C. elegans, Drosophila, Xenopus,* mice, and humans. It is expressed by virtually every cell type in most stages of development and is involved in a wide range of biological functions, including development, epithelial cell growth, carcinogenesis, and immune regulation (1). TGF-β is considered an essential component in the regulation of the immune system (2). Structurally, TGF-β belongs to a superfamily of homologous growth factors, including activin and bone morphogenic protein.

In vivo, TGF-β binds to the cell surface receptor TβRII, forming a heterodimer capable of recruiting and activating the type I receptor (TβRI). In the absence of TβRII, TGF-β has no affinity for TβRI. Upon complex formation, the constitutively active serine/threonine kinase in the cytoplasmic domain of TβRII phosphorylates the kinase domain of TβRI, thereby initiating a signaling cascade through the SMAD molecules (1,3).

TGF-β exists in five different isoforms that share 66–80% sequence identity. Forms 1–3 are found in humans, while forms 4 and 5 are found in chicken and *Xenopus,* respectively. The growth factor is expressed with an N-terminal latent peptide that is cleaved to release the 112-residue mature TGF-β. X-ray and NMR structure determination has demonstrated that TGF-β forms a disulfide-bonded homodimer having a cystine-knot structure (4–8).

TβRII is a type I transmembrane glycoprotein containing a 136-residue TGF-β-binding domain, a single transmembrane region, and an intracellular kinase domain (9). The TGF-β-binding domain contains 12 cysteine residues and forms a noncovalent homodimer.

Most TβRII expression systems use the transient transfection of mammalian cells and are not suitable for structural studies because of low yields. Until recently, the only system capable of producing a large quantity of the extracellular domain of human TβRII was the baculovirus expression system.

Bacterial expression of recombinant protein has advantages compared to other systems such as baculovirus-based or mammalian expression systems. First, bacterial-vector DNA construction is relatively simple and many existing vector systems are available. Second, bacterial expression often results in higher pro-

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[2] Abbreviations used: TGF-β, transforming growth factor β; GST, glutathione *S*-transferase; ESI-MS, electrospray ionization mass spectrometry.

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tein yields, which are necessary for structural studies. Third, the lack of posttranslational glycosylation in bacteria allows for the importance of carbohydrate to be determined and for the production of homogeneous protein samples amenable to crystallization. However, there are limitations to bacterial systems such as the production of inactive proteins. One major reason for the production of inactive proteins is the inability to form correct disulfide bonds due to a reduced cytosolic environment and a lack of thio-exchange factors. Bacterial expression of disulfide-containing proteins frequently results in the production of nonfunctional inclusion bodies requiring *in vitro* refolding to regain protein function. Certain disulfide-containing proteins, such as those with immunoglobulin-like domains that contain one or two disulfide bonds, have been successfully expressed and refolded. The production of a functional TβRII containing 12 cysteines, some or all of which participate in disulfide bonding, represents an example of producing a high cysteine content protein from a bacterial expression system.

MATERIALS AND METHODS

Deglycosylation and Protease Digestion of TβRII (Residues 1–136)

TβRII (residues 1–136) produced using the baculovirus system was deglycosylated enzymatically using peptide-*N*-glycosidase F (New England Biolabs, Beverly, MA). A mixture of 43,000 U of enzyme per milligram of receptor was incubated at 37°C for 16 h. The resulting receptor was analyzed by SDS–PAGE using a PhastSystem running a 20% polyacrylamide PhastGel (Amersham Pharmacia Biotech, Piscataway, NJ). This deglycosylated TβRII (residues 1–136) was digested using subtilisin (Boehringer Mannheim, Indianapolis, IN). A mixture of 0.002 U of enzyme per milligram of receptor was incubated at 4°C for 16 h. Subtilisin was removed using a cation exchange Poros SPM 4.6/100 column (PerSeptive Biosystems, Framingham, MA). The column was run at 1 ml/min flow rate for 10 min with 20 mM Hepes, pH 8.0, followed by a linear gradient to 1 M NaCl, 40 mM sodium citrate, pH 5.3. The flowthrough of the cation column was concentrated and then purified on a Superdex 75 HR 10/30 size exclusion chromatography column (Amersham Pharmacia Biotech) under a 0.25 ml/min flow rate in 20 mM Hepes, pH 8.0.

TGF-β-Binding Assay

Immulon4 96-well flat-bottom microtiter plates (Dynatech Laboratories, Chantilly, VA) were used for ELISAs. The samples were diluted in 0.1 M $NaHCO_3$, pH 8.6, and added at 25 μl/well. BSA (5 μg/well) was used as a blank and untreated, glycosylated TβRII (residues 1–136 at 3.2 μg/well) was used as the control. The plate was incubated at 37°C for 1 h, washed three times with distilled water, and then blocked with 3% BSA at 100 μl/well for 1-h incubation at 37°C. Reagents used were from the Quantikine human TGF-β1 quantitative colorimetric sandwich ELISA kit (R&D Systems, Minneapolis, MN).

GST/TβRII (Residues 1–136, 15–136, or 20–136) Construct in pGEX-2T

The extracellular TGF-β-binding domains of human TβRII (residues 1–136) and N-terminal truncated TβRII (residues 15–136 or 20–136) were generated using the polymerase chain reaction (PCR) and cloned into the pGEX-2T vector (Amersham Pharmacia Biotech) between the *Bam*HI and *Eco*RI restriction enzyme sites. The fusion construct consisted of an N-terminal GST, a thrombin protease cleavage site, and the TβRII (residues 1–136, 15–136, or 20–136) under the control of a *tac* promoter (Figs. 2A–C). Protein expression was carried out in *E. coli* DH5α cells (GIBCO BRL, Gaithersburg, MD). After thrombin cleavage, two residues (glycine and serine) were left at the N-terminus of the receptor. All DNA constructs were sequenced (Colorado State University, Macro Molecular Resources, Fort Collins, CO), and expressed protein was analyzed by N-terminal amino acid sequencing and ESI-MS.

His_6/GST/TβRII(K98T) (Residues 15–136) Construct in pET 15b

Mutation of Lys98 to Thr was generated by PCR of GST/TβRII (residues 15–136) and cloned into a pET 15b vector (Novagen, Madison, WI) between the *Nde*I and *Xho*I restriction enzyme sites. This construct contained a His_6-tag, a thrombin protease cleavage site, GST, a second thrombin protease cleavage site, and TβRII(K98T) (residues 15–136) under the control of the T7 promoter (Fig. 2D). Protein expression in pET vectors was carried out in *E. coli* BL21(DE3) cells (Novagen).

His_6/TβRII(K98T) (Residues 15–136) Construct in pET 15b

The pET 15b polylinker was modified by digesting the vector with the restriction enzymes *Nco*I and *Bam*HI and cloning in a synthetic (GIBCO BRL) oligonucleotide cassette (Table 1, oligonucleotides 1–4). The modified pET 15b vector contained an alanine, a His_6-tag, a thrombin protease cleavage site that included a *Bam*HI restriction enzyme site within the codons for glycine and serine, a unique *Not*I restriction enzyme site for screening of the construct, and an *Xho*I restriction enzyme site. The *Bam*HI site in wild type pET 15b

TABLE 1

Oligonucleotides used in the Construction of TβRII Expression Vectors

Number	Oligonucleotide	Construct
1	5′-catggctagcagccatcatcatcatcatcacagcagcggc-3′	Fig. 2E
2	5′-ctggtgccgcgcggatcccatatgagcggccgcctcgagt-3′	Fig. 2E
3	5′-ggatccgcgcggcaccaggccgctgctgtgatgatgatgatgatggctgctagc-3′	Fig. 2E
4	5′-gatcactcgaggcggccgctcatatg-3′	Fig. 2E
5	5′-gactcagtcagtcatatggctgtcactgacaacaacggtgc-3′	Fig. 2F
6	5′-actgactgagtcctcgagttatcagtcaggattgctggtgtt-3′	Fig. 2G
7	5′-gactcagtcagtcatatggcagtcaagtttccacaactg-3′	Fig. 2H
8	5′-gactcagtccatatggctctgtgtaaattttgtgatgtgag-3′	Figs. 2F–H

was removed. TβRII(K98T) (residues 15–136) was generated by digestion of GST/TβRII(K98T) (residues 15–136) in pET 15b with *Bam*HI and *Xho*I and then cloned into the modified pET 15b vector using the same restriction enzyme sites. The fusion construct consisted of a His_6-tag, a thrombin protease cleavage site, and TβRII(K98T) (residues 15–136) (Fig. 2E).

TβRII(K98T) (Residues 15–136, 22–136, or 27–136) Construct in pET 30a

TβRII(K98T) (residues 15–136, 22–136, or 27–136) was generated by PCR (Table 1, oligonucleotides 5–8) of TβRII(K98T) (residues 15–136) in pET 15b and cloned into a pET 30a vector (Novagen) between the *Nde*I and *Xho*I sites. This resulted in a construct containing only the TβRII(K98T) (residues 15–136, 22–136, or 27–136) (Figs. 2F–H). These constructs also contained a methionine start codon followed by an alanine and two stop codons before the *Xho*I site, which eliminated the C-terminal His_6-tag of the pET 30a vector. Bacterial expression resulted in the removal of the N-terminal methionine when a small residue such as alanine followed it. In the TβRII, residue 21 is an alanine while residue 15 is a valine and residue 26 is a glutamine.

Soluble Receptor Preparation

A 200-ml, 37°C overnight culture of *E. coli* containing the His_6/GST/TβRII (residues 15–136) pET 15b plasmid was grown in Luria–Bertani broth (LB) with 50 mg/L carbenicillin. The overnight culture was added to 10 L of Super Broth (SB) with 50 mg/L carbenicillin, 20 g/L glucose, and 0.1 ml/L antifoam in a Bioflo 3000 bioreactor (New Brunswick Scientific, Edison, NJ). The cells were grown at 37°C, 500 rpm, pH 7.0, and 6.0-psi O_2 flow until they reached an optical density at 600 nm (OD_{600}) of 3.0–5.0. The cells were induced with 50 mg/L isopropyl β-thiogalactopyranoside (IPTG) and the temperature was lowered to 30°C until the OD_{600} reached 10.0–15.0, at which time the cells were harvested.

Cells were resuspended in 600 ml of PBS containing 20 mg/L 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) protease inhibitor (Boehringer Mannheim) and lysed using a microfluidizer (Microfluidics Corp., Newton, MA). Lysozyme and DNase I were added to the lysate and stirred overnight at 4°C. Following centrifugation (25,000*g*, 40 min, 4°C), the soluble fraction was loaded onto a 10-ml glutathione–Sepharose 4B (Amersham Pharmacia Biotech) column at 2 ml/min at 4°C, washed with 300 ml of PBS, and incubated with 18 U of biotinylated thrombin (Novagen) at room temperature for 20 h. Streptavidin agarose (Novagen) was added (0.25 ml) to the supernatant to remove thrombin. The sample was concentrated using a Centriprep and Centricon 10 (Amicon/Millipore, Bedford, MA), loaded onto a Mono P HR 5/20 column (Amersham Pharmacia Biotech), and eluted with a 0–0.5 M NaCl linear gradient in 50 mM Hepes, pH 7.0. The peak from the Mono P column was further purified using a Superdex 75 HR 10/30 size exclusion chromatography column, under a 0.5 ml/min flow rate in 50 mM NaCl, 50 mM Tris, pH 8.0.

An alternative method for purifying the His_6/GST/TβRII(K98T) (residues 15–136) involved the use of Ni–NTA agarose beads (Qiagen, Valencia, CA). In brief, 40 ml of bacterial lysate containing the His_6/GST/TβRII fusion protein was loaded onto 0.5 ml of Ni–NTA agarose beads, washed with 90 ml of 5 mM imidazole, 2 M NaCl, 0.1 M Mes, pH 6.3, and eluted with 1 M imidazole.

A second alternative purification method used a polyclonal anti-GST antibody (Amersham Pharmacia Biotech). Anti-GST antibody (20 mg) was coupled to 5 ml of Protein G–Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech) using 0.25 g of dimethyl pimelimidate (Pierce Chemical Co., Rockford, IL). Bacterial supernatant (18 ml) was loaded onto the anti-GST beads, washed with 170 ml of PBS, and eluted either with 0.1 M glycine, pH 2.7, into a final concentration of 0.1 M NaCl, 0.1 M Tris, pH 9.0, or with 0.1 M triethylamine, pH 11.5, into a final concentration of 0.1 M NaCl, 0.1 M Tris, pH 8.0.

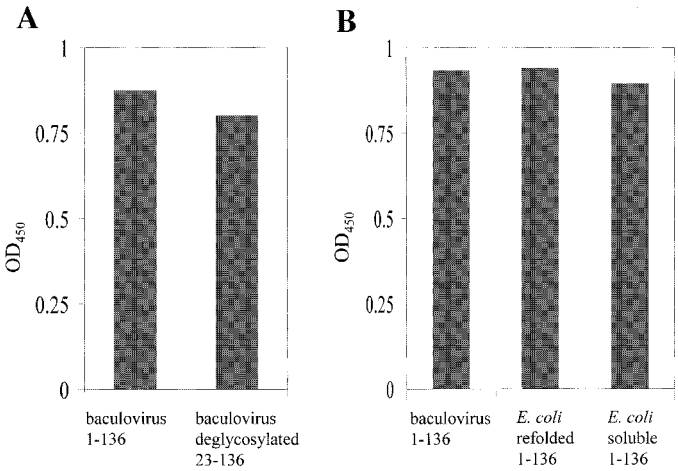


FIG. 1. Recombinant forms of TβRII retain the ability to bind their ligand, TGF-β1. (A) TGF-β1 binding of untreated baculovirus-produced TβRII (residues 1–136) compared with the binding of deglycosylated and subtilisin-treated, baculovirus-produced TβRII (residues 23–136). (B) Comparison of the TGF-β1 binding of untreated baculovirus-produced TβRII (residues 1–136), *E. coli* produced refolded TβRII (residues 1–136), and *E. coli* produced soluble TβRII (residues 1–136). The ELISA consisted of coating a microtiter plate with TβRII, binding TGF-β1, followed by binding of a conjugated anti-TGF-β1 antibody for colorimetric quantification. A well coated with BSA instead of TβRII was used as a blank.

Inclusion Body Preparation and Refolding of the Receptor

The *E. coli* expression of His_6/TβRII(K98T) (residues 15–136) in pET 15b and TβRII (residues 22–136 or 27–136) in pET 30a was performed at 37°C in a 10-L bioreactor as described above for His_6/GST/TβRII. Carbenicillin was used for the pET 15b construct and kanamycin for the pET 30a constructs. Upon harvest, cells were resuspended in 600 ml of PBS containing 20 mg/L AEBSF protease inhibitor and lysed using a microfluidizer. Lysozyme and DNase I were added to the lysate and stirred overnight at 4°C. The lysate was divided into four 200-ml centrifuge tubes and centrifuged at 25,000*g* for 40 min at 4°C. The pellet was resuspended in two 200-ml centrifuge tubes using a spatula and homogenization in 150 ml per centrifuge tube of buffer containing 1.5 M urea, 5 mM DTT, 5 mM EDTA, 0.25% Triton X-100, and 0.1 M Tris, pH 8.0. The pellet was washed by four cycles of centrifugation and resuspension with the above buffer followed by two cycles of centrifugation and resuspension with 3 mM DTT in water. The final inclusion bodies were resuspended with 3 mM DTT in water and stored at −80°C.

For refolding, 50 mg of inclusion bodies was dissolved in 10 ml of 6 M guanidine hydrochloride, 10 mM DTT, and 20 mM Tris, pH 7.0, and then injected rapidly into a 1-L solution of 0.5 M arginine, 20 mg/L AEBSF protease inhibitor, 2 mM EDTA, 5 mM cysteamine (reduced form), 0.5 mM cystamine (oxidized form), and 0.1 M Tris, pH 8.0. The solution was stirred for 16 h at 4°C and then dialyzed (3500 molecular weight cutoff) against water until the overall salt concentration was less than 10 mM.

After filtering (0.22 μm), the refolding solution was

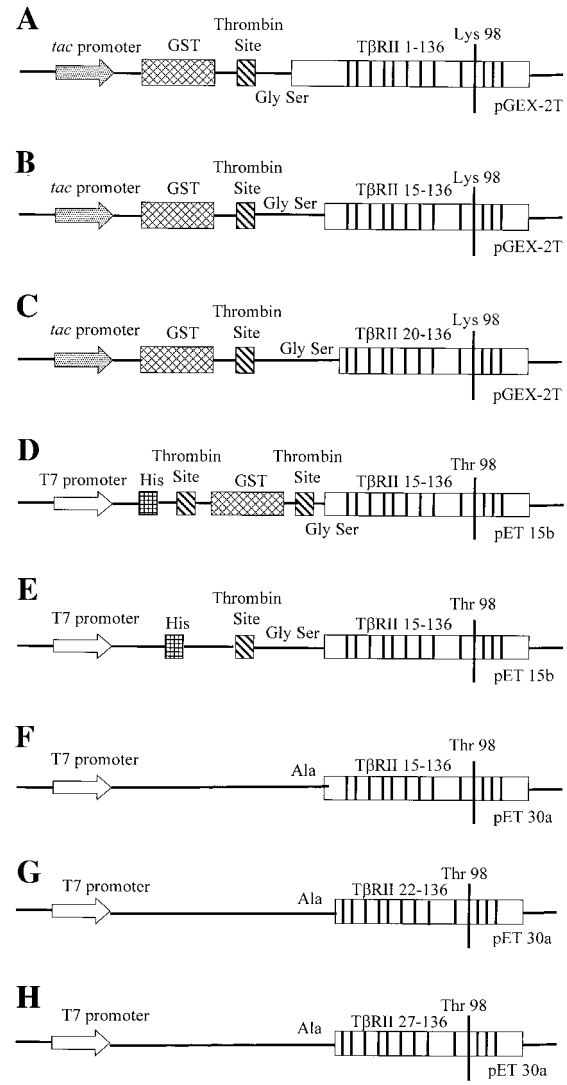


FIG. 2. TβRII expression vectors used in purification. (A–C) TβRII in a pGEX-2T vector. GST is followed by a thrombin protease cleavage site and (A) TβRII (residues 1–136), (B) TβRII (residues 15–136), or (C) TβRII (residues 20–136). After thrombin cleavage, two residues (glycine and serine) were left N-terminal to the receptor. These three receptor constructs each contained the human wild-type residue lysine at position 98. (D, E) TβRII(K98T) in a pET 15b vector. A His_6-tag is followed by a thrombin protease cleavage site and (D) GST, a thrombin protease cleavage site, and TβRII(K98T) (residues 15–136) or (E) TβRII(K98T) (residues 15–136). (F–H) TβRII(K98T) in a pET 30a vector. A methionine start codon is followed by an alanine and (F) TβRII(K98T) (residues 15–136), (G) TβRII(K98T) (residues 22–136), or (H) TβRII(K98T) (residues 27–136). Bacterial expression results in the removal of the N-terminal methionine when it is followed by a small residue such as alanine. In the TβRII, residue 21 is an alanine while residue 26 is a glutamine.

loaded onto a SOURCE 15Q column (Amersham Pharmacia Biotech) and eluted with a linear gradient from 0 to 0.75 M NaCl in 25 mM Tris, pH 8.0. The TβRII peak was concentrated to 1.0 ml, loaded onto a Mono P HR 5/20 column, and eluted with a 0–0.5 M NaCl linear gradient in 50 mM Hepes, pH 7.0. The Mono P peak was concentrated and passed through a Superdex 75 HR 10/30 column with a flow rate of 0.5 ml/min in 50 mM NaCl, 50 mM Tris, pH 8.0.

RESULTS AND DISCUSSION

When expressed using the baculovirus system, the extracellular domain of human TβRII (residues 1–136) had a molecular weight of 18 kDa as determined by SDS–PAGE. After deglycosylation with peptide-*N*-glycosidase F, the molecular weight of the receptor was 15 kDa. Further treatment of this deglycosylated receptor with subtilisin yielded a 12.5-kDa fragment lacking the N-terminal 22 amino acids. This fragment of TβRII retained the ability to bind TGF-β1 (Fig. 1A). However, enzymatic deglycosylation and proteolytic digestion were generally incomplete, resulting in a heterogeneous population of the receptor as determined by SDS–PAGE.

Since the bacterial protein glutathione *S*-transferase (GST) and human TβRII (residues 1–136) are both homodimers, a GST/TβRII fusion construct was designed to facilitate TβRII dimer formation. Expression from the pGEX-2T vector-based bacterial GST/TβRII (residues 1–136) fusion construct (Fig. 2A) produced approximately equal amounts of active receptor in both the soluble and refolded fractions. TβRII retained the ability to bind TGF-β1 (Fig. 1B). Purification of the soluble and refolded forms on glutathione–Sepharose 4B beads followed by thrombin digestion revealed that about 10% of the soluble TβRII was released compared to only about 1% of the refolded TβRII.

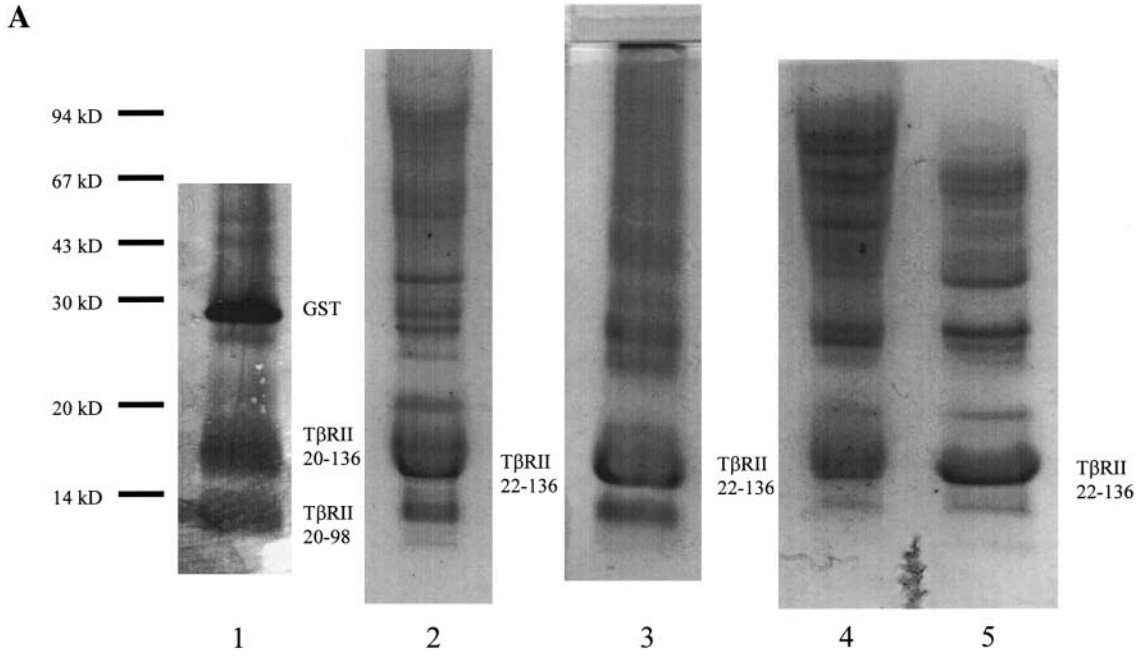
The truncated soluble GST/TβRII (residues 20–136) fusion protein (Fig. 2C) was expressed and purified using procedures similar to that of the full-length fusion construct. The thrombin-digested fusion protein revealed, however, the presence of not only free GST and TβRII (residues 20–136) but also an unknown lower molecular weight species on SDS–PAGE (Fig. 3A, lane 1). The lower molecular weight species was identified by electrospray ionization mass spectrometry (ESI-MS) as corresponding to TβRII (residues 20–98) and an additional species was identified and corresponded to residues 99–136. Similar fragments, residues 15–98 and 99–136, were also identified by ESI-MS in soluble GST/TβRII (residues 15–136) fusion protein digested by thrombin.

The presence of the C-terminal 99–136 fragment suggested a proteolytic cleavage between residues 98 and 99 rather than a premature product of RNA transcription or protein translation. Wild-type human TβRII contains lysine and cysteine at positions 98 and 99, respectively. To eliminate the degradation, Lys98 was mutated to Thr, a residue observed in chicken TβRII.

The expression of the K98T mutant receptor in the His_6/GST/TβRII(K98T) (residues 15–136) pET 15b construct (Fig. 2D) eliminated spontaneous degradation. All GST constructs yielded active but limited amounts of the receptor (10 μg of TβRII/L cell growth). Replacing the pGEX-2T vector (*tac* promoter) with the pET 15b vector (T7 promoter) resulted in a threefold increase in the expression of the GST/TβRII fusion protein. Large-scale 50-L fermentation did not improve expression yields. Due to the presence of GST and His_6-tag, the fusion protein can be purified either by glutathione–Sepharose 4B beads, by anti-GST antibody coupled to Protein G–Sepharose 4 Fast Flow beads, or by Ni–NTA agarose beads. Among these three purification methods, the best yield was obtained using glutathione–Sepharose 4B beads. To further increase the yield, GST was removed from the pET 15b construct and inclusion bodies were expressed from this His_6/TβRII(K98T) (residues 15–136) construct (Fig. 2E) and refolded. However, this construct inhibited bacterial growth.

Inclusion bodies of the receptor were 60–90% pure, making affinity purification based on the N-terminal His_6-tag unnecessary. Three new constructs without any affinity tags and with further truncations at the N-terminus were designed. The first construct, TβRII (residues 15–136) (Fig. 2F), was the same length as the GST and His_6 fusion constructs. The second construct, TβRII (residues 22–136) (Fig. 2G), was similar to the baculovirus-produced, protease-digested TβRII (resi-

FIG. 3. Analysis of TβRII purification by SDS–PAGE. (A) Lane 1: Degradation of *E. coli* produced TβRII (residues 20–136). Soluble protein expressed from the construct GST/TβRII (residues 20–136) in pGEX-2T was bound to glutathione–Sepharose 4B beads and TβRII was released by thrombin protease digestion. Lane 2: Inclusion bodies expressed from the construct TβRII(K98T) (residues 22–136) in pET 30a. Lane 3: Refolded protein expressed from the construct TβRII(K98T) (residues 22–136) in pET 30a. Lane 4: Refolded protein expressed from the construct TβRII(K98T) (residues 22–136) in pET 30a was purified on a SOURCE 15Q column. Lane 5: Mono P column peak from SOURCE 15Q peak. (B) Refolded protein expressed from the construct TβRII(K98T) (residues 22–136) in pET 30a was purified on a SOURCE 15Q column followed by a Mono P column and a size exclusion column. Superdex 75 column fractions from the TβRII peak are shown. (C) Refolded protein expressed from the construct TβRII(K98T) (residues 27–136) in pET 30a was purified on a SOURCE 15Q column followed by a Mono P column and a size exclusion column. Superdex 75 column fractions from the TβRII peak are shown.


A
94 kD
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43 kD
30 kD
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14 kD
GST
TβRII 20-136
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1
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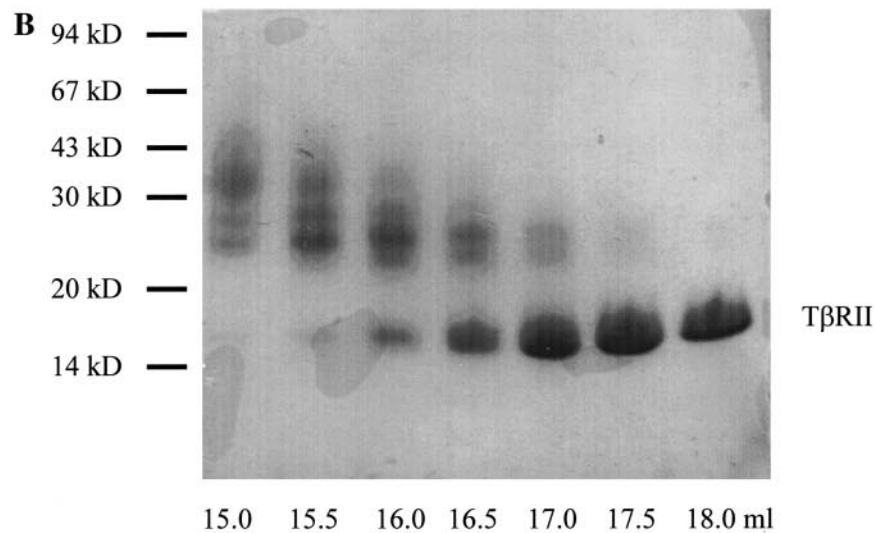
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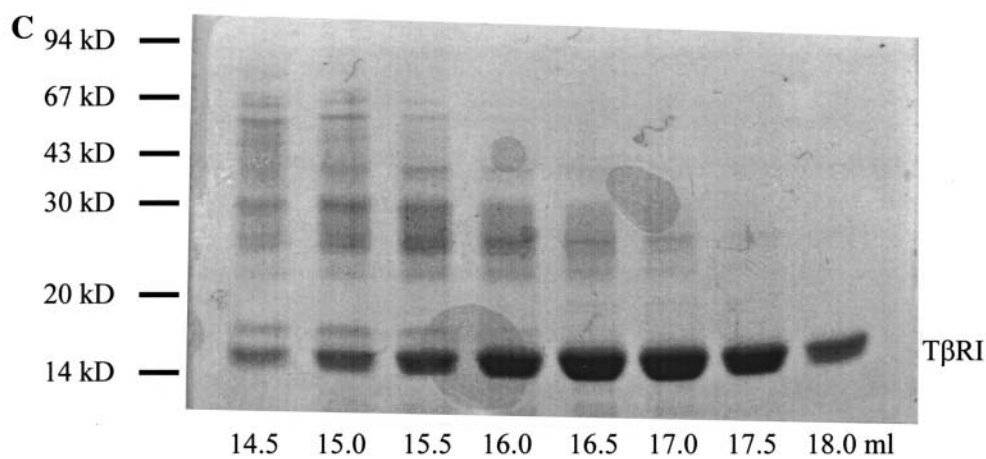
C
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TβRI
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dues 23–136). The third construct, TβRII (residues 27–136) (Fig. 2H), was created to yield a truncation up to the first cysteine residue that occurs at position 28 of TβRII. These three constructs were cloned into the pET 30a vector.

The pET 30a TβRII (residues 15–136) inhibited bacterial growth. The other pET 30a constructs each yielded approximately 500 mg of TβRII(K98T) (residues 22–136 or 27–136) inclusion bodies per 10 L growth of BL21(DE3) cells (Fig. 3A, lane 2). To refold the receptor, 50 mg of inclusion bodies was solubilized in guanidine hydrochloride and diluted into a 1-L refolding solution containing L-arginine and a cystamine redox system. This solution was dialyzed against water (Fig. 3A, lane 3) and concentrated on a strong anion exchange column (SOURCE 15Q) (Fig. 3A, lane 4), followed by Mono P column (Fig. 3A, lane 5) and size exclusion chromatography (Figs. 3B and 3C). The yield of active TβRII(K98T) was 5 mg.

In summary, a total of four constructs were made to express four different forms of TβRII. A K98T mutation was made to eliminate an internal proteolytic site. The removal of up to 26 N-terminal residues retained binding to TGF-β1. TβRII expressed as a fusion protein with GST was produced in a soluble, active form but with a low yield. TβRII expressed alone was found in inclusion bodies and required *in vitro* reconstitution to regain activity. The final yield of functional receptor from inclusion bodies was approximately 500 times higher than from the GST fusions.

Recently, the soluble extracellular domain of the mouse type II activin receptor, also a member of the TGF-β receptor superfamily, was expressed in yeast and crystallized (10,11). *Escherichia coli* expression of C-terminal truncated human TβRII (residues 3–114), on a 50-ml scale, was also reported to retain neutralizing activity as measured by a NOB/CTLL bioassay (12). However, this reported TβRII (residues 3–114) construct lacks two C-terminal cysteine residues, Cys115 and Cys120.

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